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Research Article

In Vitro and *in Vivo* Analysis of the Antioxidant Potentials of Methanolic Extract of *Tamarindus indica* Leaves

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ABSTRACT

This study evaluated the antioxidant potentials of methanolic extract of *Tamarindus indica* leaves using *in vitro* and *in vivo* assays. Preliminary qualitative screening of phytochemicals revealed the presence of alkaloids, flavonoids, saponins, tannins, cardiac glycosides and steroids. The result of the acute toxicity test of the extracts revealed the LD_{50} to be above 5000 mg/kg through both oral and intra-peritoneal routes. Both the *in vitro* and *in vivo* assays were carried out. The reducing power (0.45±0.007 µg/ml) was recorded for *T. indica* with a difference of 0.77% in comparison to the control and DPPH value (68.10 ± 0.007%) with a significant increase compared to ascorbic acid. The *in vivo* analysis shows that lipid peroxidation, superoxide dismutase and catalase activities have values of 22.75 ± 9.29 nmol/mg, 1.60 ± 0.03 U/ml and 0.003±0.001 µmol/mg, respectively. The values for superoxide dismutase and lipid peroxidation were significantly different compared to the control (1.48 ± 0.03 U/ml and 24.54 ± 9.29 nmol/mg, respectively) while the value for catalase activity was not significant compared to the control (0.0025 ± 0.001 µmol/mg). The liquid chromatography mass spectrometry revealed the presence of some important metabolite; linoleic acid and quassin, among others, which are antioxidant. Based on the results obtained, the *T. indica* leaves contain vital metabolites with antioxidant potential that could be useful in health management.

Keywords: *Tamarindus indica, Antioxidant, LCMS, Phytochemicals, Free radical*

INTRODUCTION

Since ancient times, medicinal plants, fruits, and vegetables are well known to be rich in biologically active compounds, which make them a prospective source of therapeutic agents (Oyewole and Kalejaiye, 2012). Presently, natural products are used in several developments of new drugs for the treatment of chronic disease (Borokini *et al.*, 2017). The tradition of using plants for the treatment of wounds, fever, malaria, among others, is existing for many years (Meher and Dash, 2013). Free radical molecules most especially reactive oxygen species (ROS) are generally derived from biotransformation of molecular oxygen (Banjarnahor and Artanti 2014). The commonly known ROS, include superoxide anion radical (O^{2-}), singlet oxygen (O_2), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical (OH^-). The detrimental effects of oxygen are linked to its metabolic reduction to these highly toxic species. Free radicals lead to oxidative damage often referred to oxidative stress, and has been associated with several degenerative diseases, such as cancer, osteoarthritis, diabetes, cardiovascular diseases, etc. Alteration of critical balance between ROS production and endogenous antioxidant defense mechanism lead to oxidative stress (Banjarnahor and Artanti, 2014).

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Tamarindus indica belongs to the Dicotyledous family and leguminose sub family Caesalpiniaceae, which is the third

largest family of flowering plants with a total of 727 genera and 19,327 species (Lewis *et al.,* 2005). Tamarind (*Tamarindus indica*) is increasingly becoming a commercially important underutilized tree crop worldwide. Due to its multi-purpose use and market demand the tree and its processed products are been traded in many part of the world (Chimsah *et al*., 2020). Extracts from plants have been used among traditional healers, especially in tropical areas where there are plentiful sources; therefore the assessment of anti-oxidative properties will not be over emphasis. This study will investigate the *in vivo* and *in vitro* antioxidant potential of *T. indica* methanol leaves extract and identify the possible bioactive compound using liquid chromatography mass spectrometer.

MATERIALS AND METHODS

Chemicals/Equipment

Solvent/reagents used were purchased from Sigma Aldrich and were of analytical grade, Liquid chromatography-mass spectroscopy (LC Water e2695 separation module with W2998 PDA and couple to ACQ-QDA MS). UV-VIS Spectrometer (Agilent Carry 300), Centrifuge Weighing balance, water bath, and pH meter were used.

Sample collection/ preparation

Leaves of *T. indica* were collected from Kano State, Nigeria. The leaves were identified at the herbarium unit of Bayero University, Kano, with the voucher number BUKHAN 74. Wistar albino rats were obtained from Department of Pharmacology, Faculty of Pharmaceutical Sciences, Ahmadu Bello University, ABU, Zaria, Kaduna State, Nigeria. The leaves were shade dried at room temperature (25^0C) for two weeks and pulverized into a coarse powder using clean mortar and pestle. The coarse powder was weighed, labelled and kept in an airtight container for further analysis.

Extraction

Successive extraction was carried out by cold maceration procedure as reported by Sharif *et al.* (2017) using three different solvents viz; n-hexane, ethyl acetate and methanol. The leaves powder (40 g) was dissolved in 200 cm^3 of the solvent. The mixture was allowed for 48 hours with frequent intermittent agitation after which the mixture was filtered using filter paper (Whatman No. 1). The filtrate was then concentrated on steam bath to evaporate and the crude extract was obtained. Qualitative phytochemical screening test was carried out, and methanol extract showed the presence of more secondary metabolites in comparison with the others. This necessitated the use of methanol crude extract to carry out the analysis of this study.

Experimental animals

Sixteen male Wistar albino rats weighing between 100 and 250 g were used for acute toxicity studies. Twenty male Wistar albino rats weighing between 100 and 250 g were kept in a well-ventilated aluminium cage at room temperature and under natural light/darkness cycles in the laboratory were used for *in vivo* analysis. The animals were allowed access to feed and water *ad libitum* and allowed two weeks to acclimatize before the commencement of the experiment. The animals were maintained in accordance with the recommendation of the Guide for the care and use of laboratory animals (Department of Health & Human Resources, DHHS, 1985).

Acute toxicity studies (Determination of LD_{50})

Acute toxicity study of *T. indica* leaves methanol extract was carried out to determine the LD_{50}. The procedure involves two phases. The acute toxicity (LD_{50}) was estimated both orally and intra-peritoneally in rats (n=16) in each case following Lorke's method (Lorke, 1983). The rats were weighed and grouped by randomizing method into four groups of three rats each. The first group was the control group. Dose levels of 10, 100, and 1000 mg/kg were used for the first phase (Sharif, 2015). The number of deaths in each group within 24 hours was recorded. In the second phase, which will be deduced from the first phase, four rats were grouped into four groups of one rat each and they will be treated with doses of 1200 mg/kg, 1600 mg/kg, 2900 mg/kg, and 5000 mg/kg orally and intra-peritoneally. They were also observed for 24 hours as in the first phase, and final LD50 value was determined from Lorke's formula as follows:

$$LD_{50} = \sqrt{(a \times b)}$$

where, a is the highest dose at which no death occurred in the second phase and b is the least dosage at which death occurred in the second phase (Oyewole *et al.,* 2013). The extract was classified using the LD_{50}.

DPPH assay

This assay was carried out as described by Shen *et al.* (2010) with slight modification. Briefly, 0.1 mM solution of DPPH in methanol was prepared and 2 ml of this solution was added to 1 ml of various concentration of the extract fraction (0.16, 0.3125, 0.62, 1.25 and 2.50 mg/ml).The mixtures were shaken vigorously and incubated at room temperature for 30 minutes. Methanol was used as a blank and DPPH in methanol without the plant extract was used as positive control. Then the absorbance was measured at 517 nm using a UV-VIS Spectrophotometer. Ascorbic acid was used as the reference. The capability of scavenging the DPPH radical

was calculated by using the following formula; DPPH scavenging effect

$$\% \text{ inhibition of DPPH radical} = \frac{\text{Abr} - \text{Aar}}{\text{Abr}} \times 100$$

Where, Abr is the absorbency before reaction and Aar is the absorbency after reaction has taken place.
Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicate. The IC_{50} value was determined as the half-maximum concentration of the extract that scavenges 50% of DPPH.

Ferric reducing power assay
The reducing power of *T. indica* leaves methanol extract was measured using the method described by Ferreira *et al.* (2007) with slight modification. Briefly, 2.5 ml of extract fractions at different concentrations (0.1625, 0.625, 0.3125, 1.25 and 2.50 mg/ml) were mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixtures were incubated at 50°C for 20 minute, and allowed to cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 minute. The supernatant (2.5 ml) for each were mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 0.1%) then finally allowed to stand for 10 minute. The absorbance was read at 700 nm using UV-Vis spectrometer. Ascorbic acid was used as standard against reagent in distilled water as blank.

***In vivo* anti-oxidative potentials of *Tamarindus indica* leaves methanol extract**
Twenty male albino Wistar rats were randomly divided into four groups of five animals each. Group 1 served as the control and receive 0.4 ml of distilled water. Group 2 received 100 mg/kg of the extract. Group 3 received 200 mg/kg of the extract, and group 4 received 400 mg/kg of the extract (Samuel *et al.,* 2014). The animals were dosed daily for 21 days and observed daily for changes and other signs of toxicity such as excessive salivation, change of eye colour as well as death throughout the period of study.

Serum preparation
Twenty-four hours after the last treatment, blood was obtained through direct cardiac puncture which was used to assay for *in vivo* antioxidant activity of the plants extracts (Samuel *et al.,* 2014). The blood was preserved in plain bottles. The serum was prepared using the standard method as described by Yesufu *et al*. (2010). Blood was allowed to clot for 30 minutes and then centrifuged at 2500 rpm for 15 minutes and then the serum was collected.

Determination of the lipid peroxidation (LPO) in serum
Lipid peroxidation was estimated by measuring malondialdehyde (MDA) as described by the method of Albro *et al.* (1986). About 0.1 ml of serum was treated with 2 ml of (1:1:1 ratio) TBA–TCA–HCL reagent (TBA 0.37%, 0.25 N HCL and 15% TCA) and placed in water bath for 15 minutes, cooled and centrifuged. Absorbance of a clear supernatant was read at 535 nm.

Estimation of superoxide dismutase (SOD)
Superoxide dismutase (SOD) was determined using the method described by Fridovich (1989). The ability of superoxide dismutase (SOD) to inhibit auto-oxidation of adrenaline at pH 10.2 formed the basis of this assay. Carbonate buffer (0.05 M) which comprises of 114.3 g of Na_2CO_3 and 4.2 g of $NaHCO_3$ was dissolved in distilled water and was made up to 1000 ml in a volumetric flask. Adrenaline (0.3 mM): 0.01 g of adrenaline was dissolved in 17 ml of distilled water; the solution was prepared fresh. The serum of 0.1 ml was diluted in 0.9 ml of distilled water to make 1:10 dilution of the microsome. An aliquant mixture of 0.2 ml of the diluted microsome was added to 2.5 ml of 0.05 M carbonate buffer. The reaction was initiated with the addition of 0.3 ml of 0.3 mM Adrenaline. The reference mixture contained 2.5 ml of 0.05 M carbonate buffer, 0.3 ml of 0.3 mM Adrenaline as well as 0.2 ml of distilled water. The absorbance was measured from 30 seconds up to 150 seconds at 480 nm.

$$\text{Increase in absorbance per minute} = \frac{(\text{A2} - \text{A1})}{2.5}$$

Where, A2 is the absorbance recorded at 150 secs and A1 is the absorbance recorded at 30 secs

$$\% \text{ Inhibition} = 100 - \frac{\text{Increase in absorbance for sample}}{\text{Increase in absorbance of blank}} \times 100$$

One unit of SOD activity is the quantity of SOD necessary to elicit 50 % inhibition of the oxidation of Adrenaline to adenochrome in 1 minute.

Estimation of catalase activity
Catalase (CAT) activity was measured using the Abebi (1974) method. Exactly 10 µl of serum was added to a test tube containing 2.80 ml of 50 mM potassium phosphate buffer (pH 7.0). The reaction was initiated by adding 0.1 ml of freshly prepared 30 mM H_2O_2 and the decomposition rate of H_2O_2 was measured at 240 nm for 5 minutes. A molar extinction coefficient (E) of 0.041 mM^{-1} cm^{-1} was used to calculate the catalase activity.

$$\text{Catalase Activity} = \frac{\text{Catalase Concentration}}{\text{Protein Concentration (mg/ml)}}$$

Determination of protein

The total protein content of the serum was assayed using Biuret method.

LC/MS Analysis

The samples were analyzed using liquid chromatography (LC) tandem mass spectrophotometry (MS) as described by Piovesana, *et al.,* (2019) with some modifications. The extracted sample was reconstituted in LCMS grade methanol and filtered through polytetrafluoroethylene (PTFE) membrane filter with 0.45 µm size. The filtrate (10.0 µl) was injected into the LC system and allowed to separate on Sunfire C18 (5.0 µm 4.6 mm x 150 mm) column. The run was carried out at a flow rate of 1.0 mL/min, Sample and Column temperature at 25 ^{0}C. The mobile phase consists of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) with a gradient (Table 1)..

Table 1. Solvent Gradient of 0.1% Formic Acid in Water (solvent A) and 0.1% Formic Acid in Acetonitrile (solvent B)

Time (S)	Solvent A (%)	Solvent B (%)
0	95	5
1	95	5
13	5	95
15	5	95
17	95	5
19	95	5
20	95	5

From ratio of A/B 95:5 this ratio was maintained for further 1 min, then A/B 5:95 for 13 min to 15 min. then A/B 95:5 to 17 min, 19 min and finally 20 min. The PDA detector was set at 210-400 nm with resolution of 1.2 nm and sampling rate at 10 points/sec. The mass spectra were acquired with a scan range from m/z 100–1250 after ensuring the following settings: ESI source in positive and negative ion modes; capillary voltage 0.8 kV (positive) and 0.8 kV (negative); probe temperature 600^{0}C; flow rate 10 ml/min; nebulizer gas, 45 psi. MS set in automatic mode applying fragmentation voltage of 125 V. The data was processed with Empower 3. The compounds were identified on the basis of the following information: fragmentation pattern, base m/z and compared with data base in SDBS data base for organic compound.

RESULTS

Phytochemical screening

Phytochemical screening for the bioactive metabolites present in methanol crude extracts of *T. indica* leaves is presented in Table 2. The phytoconstituents evaluated include alkaloids, flavonoids, saponins, tannins, cardiac glycosides, anthraquinones and steroids. The result of the phytochemical screening shows that, methanol crude extract contains more secondary metabolites.

Table 2: Qualitative Phytochemical Screening of *Tamarindus indica* Methanol Leaves Extract

Phytoconstituents	T. I
Alkaloids	+
Steroids/ Triterpenes	+
Cardiac Glycosides	+
Anthraquinones	-
Tannins	+
Flavonoids	+
Saponin	+

(+) indicates presence and (-) indicates absence; T.I. - *Tamarindus indica*

Acute toxicity test

Acute toxicity test of *T. indica* extract revealed the LD_{50} to be above 5000 mg/kg through both oral and intra-peritoneal route of administration (Table 3).

Table 3. Acute Toxicity Test Results of *T. indica* Leaves Methanol Extract (Orally and Intra-peritoneally)

Dose (mg/kg)	First Phase (Death/Survival) *Tamarindus indica*
10	0/3
100	0/3
1000	0/3
	Second Phase (Death/Survival)
600	-
1000	-
1600	0/1
2900	0/1
5000	0/1

***In vitro* and *in vivo* assays**

The antioxidant potentials of *T. indica* leaves methanol extract were presented in Tables 4 and 5 for *in vitro* and *in vivo* assays, respectively. Results from *in vitro* assay revealed significant ($p < 0.05$) difference between the extract and control and with their concentration on reducing power and DPPH activities. The reducing power activity (0.45) was recorded for *T. indica* with a difference of 0.77 % in comparison to the control. Results on DPPH activity showed significance difference when compared to the control. There was a decreasing trend in concentration on reducing power and DPPH activity of *T. indica* leaves extract i.e. from 1000 µg/ml which decreased progressively to 15.625 µg/ml. The concentration (serial dilution) effect on *T. indica* leaves extract shows that a specific concentration is optimum for achieving reducing power and DPPH activity. One thousand 1000 µg/ml is optimum for reducing power activity while for DPPH activity there was no statistical difference observed between 1000, 500 and 250 µg/ml concentration levels used.

Table 4. *In Vitro* Assays using Reducing Power and DPPH Activity of *T. indica* Leaves Methanol Extract

Extract	Reducing Power (µg/ml)	α, α-diphenyl-β-picrylhydrazyl (DPPH) (%)
Tamarindus indica	0.45^c	68.1^b
Control	0.43^d	88.2^a
SE±	0.007	0.007

[a, b, c, d] means in the same row with different superscripts are significantly different ($p < 0.05$)

Table 5 shows the results obtained from the *in vivo* assay where parameters such as lipid peroxidation, superoxide dismutase and catalase activities were shown. The *T. indica* leaves extract at 100, 200 and 400 mg/ml used in this study produced significant ($p < 0.05$) effect on lipid peroxidation and superoxide dismutase (SOD) and did not produce significant ($p > 0.05$) variations in catalase activity.

LC/MS profile of *Tamarindus indica* leaves *methanol* extract

The LCMS profiling of the extract is presented in Table 6. The tentative biomolecules or metabolites were identified by comparing their molecular fragmentation pattern obtained from the MS with those in data base. However, the identified compounds were quassin and linoleic acid and their molecular fragmentation were presented in Figures 1 and 2, respectively.

Table 5. The Effect of *T. indica* Leaves Methanol Extract on Lipid Peroxidation, Superoxide Dismutase and Catalase Activity in Wistar Rats using *In Vivo* Assay

Extract	Lipid Peroxidation (nmol/mg)	Superoxide Dismutase (U/ml)	Catalase Activity (µmol/mg)
Tamarindus indica	22.75^c	1.60^a	0.003
Control	24.54^c	1.48^b	0.0025
SE±	9.29	0.03	0.0008

[a, b, c] means in the same row with different superscripts are significantly different ($p < 0.05$)

Table 6. LC/MS Profile of *T. indica* Leaves Methanol Extract

Peak	Tentative Compound	MW (g/mol)	Precursor Ion (m/z)	Product ion (m/z)	Analyzer/ Ionization Mode
1.	Linoleic acid	280	281.512	104.287, 118.136, 227.410	QqQ/ESI (+)
2.	Quassin	388	389.445	265.256, 321.863, 337.916	QqQ/ESI (+)

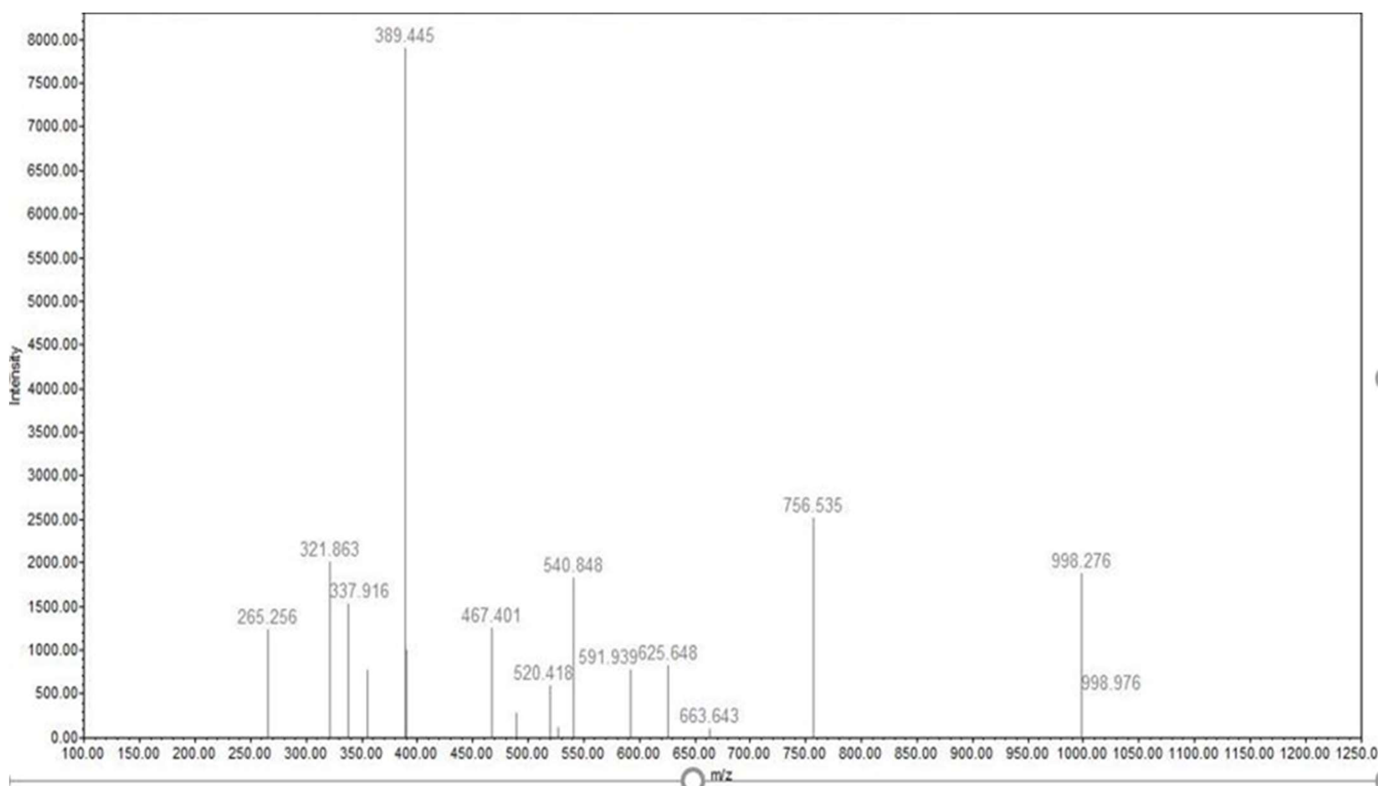

Figure 1. Fragmentation Pattern of Quassin from *T. indica* Leaves Methanol Extract

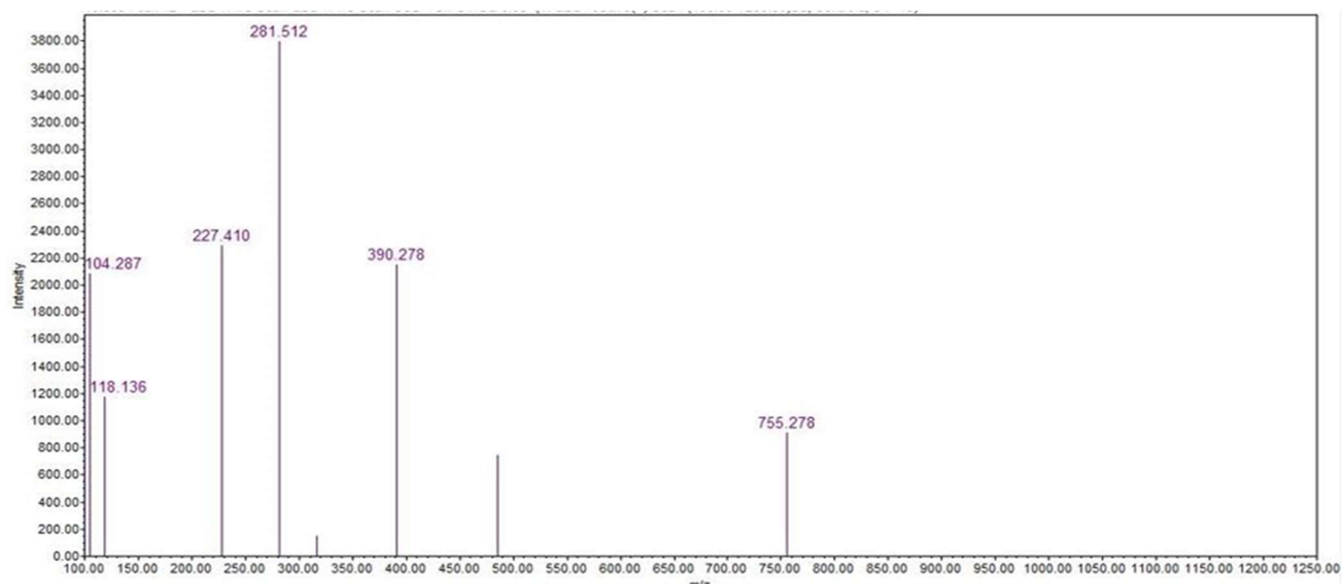

Figure 2. Fragmentation Pattern of Linoleic Acid from *T. indica* Leaves Methanol Extract

DISCUSSION

The acute toxicity test of *T. indica* leaves methanol extract revealed that the LD_{50} is above 5000 mg/kg through both oral and intra-peritoneal routes of administration. The experimental animals did not show any toxicological signs such as writhing, diarrhoea, depression, hypermotility and aggression compared to the control which is an indication of its safety for consumption as reported previously (Bako *et al.*, 2014; Sharif *et al.*, 2017). This finding is in conformity with previous studies of Abubakar *et al.* (2008) wherein behavioural changes such as fatigue was observed and it could be attributed to the presence of secondary metabolites in the extract such as saponin.

The mechanism of antioxidant action *in vitro* involves direct inhibition of the generation of reactive oxygen species or scavenging the free radicals and ability of compounds to reduce DPPH radical via hydrogen donating forming a yellowish non-radical form of DPPH-H (Hwang and Thi, 2014). The extract used in this assay showed high DPPH radical scavenging activity of 68.10 ± 0.007% which is relatively similar to many studies previously reported (Kankara and Go, 2016) and this could be as a result of its high content of phenolic compounds. Many phenolic compounds have been reported to possess potent antioxidant activity, which vary according to the number and position of hydroxyl groups. Comparison among the different classes of phenolic compounds showed that tannins have the best potential towards DPPH radical scavenging effect (Meher and Dash, 2013). The findings of the current study are consistent with those of Bendiabdellah *et al.* (2012) who reported that methanol extract of *Daucus crinitus* exhibited the highest DPPH scavenging compared to water extract.

The phytochemical studies carried out on the extracts revealed the presence of tannins, flavonoids, alkaloids, triterpenes, saponins, cardiac glycosides and anthraquinones, which were previously reported to be excellent antioxidants (Ramanathan *et al.*, 2003). Barku and Abban (2013) reported that methanol extract was found to contain greater number of metabolites than the ethyl acetate and n-hexane extracts.

An increased level of serum enzymes with decreased lipid peroxidation were observed in this study, which could be as a result of the ability of medicinal plant extract to restore the activities of these serum enzymes and scavenge the free radicals (Cotello, 2001). Also, other literature reported that this might be attributed to the presence of phytochemical constituents such as phenols, flavonoids, triterpenes, and steroids in the medicinal plants (Dai and Mumper, 2010).

Numbers of studies reported in literature have indicated that flavonoid intake gives positive effect on cardiac performance (Banjarnahor and Artanti, 2014). Recently, *in vivo* studies conducted by Liu *et al.* (2014) using experimental rat found that quercetin increases the production of antioxidant enzyme, such as glutathione-peroxidase (GSH-Px), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT). Furthermore, the authors reported that quercetin helps to preserve membrane integrity and subsequently prevents lipid peroxidation. Therefore, the extract could be a good supplement that will help in prevention of lipid peroxidation and subsequently organ damage.

The result of the LC-MS analysis of the *T. indica* leaves methanol extract revealed many metabolites with

pharmacological benefits. The compounds identified among others are quassin and linoleic acid. Quassin is a triterpene and has been used as antihypertensive, antioxidant, anti-inflammatory and anti-proliferative agent as reported by Kamarulzaman *et al.* (2017). Therefore, based on this metabolite identified from *T. indica* leaves methanol extract, it could be considered as a natural alternative to antioxidants in metabolic disorder in which level of free radical increased.

Lamiaceae herbs are known to contain high concentrations of phenolic compounds and other antioxidants (Ahn *et al.*, 2020). In a study where the total phenolic content and antioxidant properties of 23 culinary and medicinal herbs were examined, the Lamiaceae genus had a major effect on phenolic concentrations, FRAP reducing and DPPH antioxidant abilities. Therefore many literatures reported the antioxidant potential of some other medicinal plants like *Samadera indica* due to the existence of tri-terpenoids; quassinoids, according to Vidya *et al.* (2011). Thus, it is implied from the extracts of these plants, a potent antioxidant can be extracted and established, which can be used as a natural antioxidant.

Another important compound identified by the LCMS profiling from this plant extract is linoleic acid, a very useful organic acid that contributed to the activity observed in this study. Also, in a study reported by Débora and Neuza (2011), *T. indica* contains a larger proportion of unsaturated fatty acids, particularly linoleic acid (59.61%), which is consistent with the findings of this current investigation. Adeyeye *et al*. (2014) discovered that *Zingiber officinale* (ginger) has a fatty acids profile with 65.4% polyunsaturated fatty acids and 17.5% saturated fatty acids with linoleic acid accounting for 52.7% of the total fatty acids composition. The good antioxidant capacity of *T. indica* and *Zingiber officinale* (ginger) could be linked to the presence of linoleic acid.

CONCLUSION

In conclusion, this study showed the antioxidant potentials of methanolic extract of *T. indica* leaves, which could be as a result of the presence of prominent metabolites identified by LCMS analysis (quassin and linoleic acid). Therefore, it is suggested that *T. indica* leaves could be useful as a potential means for treatment of oxidative stress related diseases, pharmacological benefits and drug development in general.

AUTHORS' CONTRIBUTIONS

The conceptualization of the study was by author YG. Authors NUB and SYM drafted and revised the manuscript. Formal analysis was done by authors NUB and MB; Investigation and Methodology by NUB and MB. All the authors read through the final version and gave approval for its publication.

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None

CONFLICT OF INTEREST

The authors wish to declare that there is no conflict of interest.

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